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(54) Title: SURVIVIN, AND PEPTIDES THEREOF, AS AN ANTI–CANCER VACCINE

(57) Abstract

Vaccines capable of eliciting an antitumor response for a wide spectrum of cancers are disclosed. The active ingredient in such vaccines is provided, for example, as an antigenic material that comprises one or more epitopes of the protein survivin, a recombinant expression system capable of expressing survivin antigen for administration to the tissues of said subject; a DNA molecule that comprises an encoding sequence for survivin antigen, capable of expression of said antigen in the cells of a subject; or an antiidiotypic antibody, or fragment thereof, which mimics said antigen.

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# Survivin, and Peptides thereof, as an Anti-Cancer Vaccine

## Field of the Invention

The present invention is directed to the field of cancer treatment and prevention. More specifically, the invention concerns the use of tumor-associated antigens, recombinant expression systems for tumor-associated antigens, and antiidiotypic antibodies bearing an image of such antigens, all of which are useful in the production of vaccines to induce, in a patient, and immune response useful in the prevention or treatment of a wide variety of cancers. The invention is more particularly related to B-cell and T-cell mediated immune responses against the tumor-specific protein survivin.

## Reported Developments

Cancer cells are defined as cells that have become malignantly transformed, so that they exhibit uncontrolled growth and invasive properties. Depending on the cell type involved, cancer cells may form a solid tumor, or in the case of blood cells, present as a leukemia. In addition to the defining traits of uncontrolled proliferation and capacity to metastasize (that is, to invade other tissues), cancer cells typically exhibit numerous other differences from normal cells. For example, cancer cells may appear, morphologically, not unlike undifferentiated embryonic cells, and may express surface proteins normally found only in embryonic or fetal cells. As with embryonic cells, cancer cells may have the capacity to trigger local angiogenesis, that is, they can recruit their own blood supply by stimulating vessel cell development.

As aforementioned, cancer cells may share numerous features such as cell surface markers with embyonic cells. Additionally many oncogenes, for example viral genes which promote tumor development in a host, are closely related to (and probably evolved from) genes which function during normal embryogenesis in the host to encode proteins such as growth factors and transcription factors. Accordingly, it can be seen that one of the great difficulties in treating cancers is that such cells are in many ways normal, and are not defined by abnormalities susceptible to therapeutic intervention. For example, although the immune system is capable of distinguishing self cells from invading foreign cells, or from infected self cells that express

components from the infecting agent, a typical cancer cell may present to the immune system no molecular differences that are recognized as foreign, thereby avoiding immune system detection and killing. Additionally, most pharmaceuticals that are toxic to tumor cells are also toxic to normal body cells thereby severely limiting the application of such substances. Accordingly, there is a considerable need to identify in tumor cells additional characteristics that not only clearly distinguish such cells from normal body cells, but which also allow for successful intervention to disable or kill the tumor cells.

One area of investigation that is very promising in this regard relates to cell apoptosis. Apoptosis is a programmed form of cell death that is believed to be under direct genetic control. As such, it is different from degenerative processes such as necrosis. During apoptosis cells lose there cell junctions, the nucleus fragments and cytoplasmic components become compacted. The affected cell ultimately breaks up into vesicles which are typically absorbed by surrounding cells.

Cell apoptosis is of considerable importance in relation to tissue homeostasis, during tissue development. Inappropriately increased or decreased apoptosis is believed to contribute to many disease states, including, for example, autoimmune disorders. Recently, a considerable number of proteins that regulate apoptosis have been identified [for example, see, M. Rothe et al., *Cell*, 83, 1243-1252, 1995; N. Roy et al., *Cell*, 80, pp.167-178, 1995; C. Duckett et al., *EMBO Journal*, 15, pp. 2685-2694, 1996; M. Birnbaum et al., *J. Virology*, 68, pp.2521-2528, 1994]. It has also become apparent that disregulation of apoptosis (resulting in suppression of cell death) contributes to the development of various cancers (see C.Thompson et al., *Science*, 267, pp.1456-1462, 1995; P.Liston et al., *Nature*, 379, pp. 349-353, 1996).

Recently, an additional mammalian apoptosis inhibitor protein, named survivin, has been discovered and characterized (see international patent application WO 98/22589, published May 28, 1998, of D. Altieri, entitled "*Survivin, a protein that inhibits cellular apoptosis, and its modulation.*"; G. Ambrosini et al., *Nature Medicine*, 3, pp.917-921, 1997; Adida et al., *American Journal of Pathology*, 152, pp. 43-49, 1998; G. Cirino et al., *J. Clinical Investigations*, 99, pp. 2446-2451, 1997; and C. Adida et al., *The Lancet*, 351, pp. 882-883, 1998). Survivin was determined to be a small (16.5 kiloDalton) cytoplasmic protein that contains structural features in

common with other apoptosis inhibiting proteins. Quite interestingly, the gene for survivin was determined to be very much homologous to that of another protein investigated by those authors, of apparently unrelated function, termed EPR-1. It appears that the two proteins are encoded by separate nucleotide sequences, thus suggesting a gene duplication event leading to a head-to head configuration. Thus the two proteins are apparently coded from opposite strands of the involved chromosome. Accordingly, it is possible that the two encoding nucleotide sequences are involved in natural antisense regulation of the expression and production of the other protein.

Survivin was detected (see the above references) in mammalian cells during embryonic and fetal development, but was undetectable in terminally differentiated adult tissues. However, those authors also determined (see particularly G. Ambrosini et al., above) that survivin becomes predominantly expressed in cells representing transformed cell lines, and in all of the most common human cancers of lung, colon, pancreas, prostate and breast. Survivin was also detected in a very significant percentage of lymphomas. Accordingly, those authors concluded that apoptosis inhibition is a general feature of cancer, and determined that survivin is a likely target from which to design *apoptosis-based* cancer therapies (G. Ambrosini et al., at 917, 920).

The coding strand for survivin (referencing also the complementary transcript for EPR-1) was determined to comprise an open reading frame predicting a novel protein of 142 amino acids, unrelated to EPR-1, with a predicted molecular weight of 16,389 and a isoelectric pK of 5.74. The resultant amino acid sequence was predicted to lack an amino terminal signal peptide for secretion or a carboxy-terminal hydrophobic stretch sequence for membrane insertion. Accordingly, the protein is apparently intracellular in its expression pattern. Given that survivin is primarily only expressed in the cancer cells of subjects, it is immediately, and hopefully, a possible target for one or more cancer therapy approaches. However, given that survivin is not a membrane or secreted protein, but is only expressed intracellularly, survivin has apparently not been considered a likely target for various forms of immunotherapy including, for example, vaccine administration.

## Summary of the Invention

According to the practice of the present invention, it is recognized that mechanisms exist to cause detection by a subject's immune system of cells that express survivin, and thus subjects afflicted with cancer, or at risk therefor, can be successfully vaccinated, with resultant immune inactivation/killing of cancerous or pre-cancerous cells. In a further preferred embodiment, the subject is a cancer patient at risk for recurrence following removal by surgical, or other means, of a tumor.

Accordingly, there is provided a vaccine for eliciting an antitumor immune response in a subject which comprises an active ingredient selected from the group consisting of:

(a) an antigen that comprises one or more epitopes of survivin protein;

(b) a recombinant expression system capable of expressing survivin antigen for administration to the tissues of said subject;

(c) a DNA molecule that comprises an encoding sequence for survivin antigen, capable of expression of said antigen in the cells of a subject; and

(d) an antiidiotypic antibody, or fragment thereof, which mimics said antigen.

In preferred examples thereof, the vaccine further comprises at least one adjuvant capable of enhancing said antitumor immune response, and said antigen is optionally encapsulated in, or coupled to, a liposome.

The invention also provides for a method to induce an antitumor immune response in a potential or tumor-bearing subject, which method comprises administering to said subject a vaccine of the invention, wherein said subject is a human or veterinary patient.

Further aspects of the invention are described according to the detailed description of the invention, which follows directly.

## Detailed Description of the Invention

As aforementioned, according to the practice of the present invention, it is recognized that mechanisms exist to cause detection by a subject's immune system of cells that express survivin, in spite of survivin's predicted intracellular location, and thus subjects afflicted with cancer, or at risk therefor, can be successfully vaccinated. Prior to describing the various vaccine compositions and methods of the invention,

there is first described a brief mention of the immune system components whose nature and function determines, in part, how the present invention can be practiced.

According to the practice of the present invention, it is recognized that cellular processes that involve both major histocompatibility complex (MHC)-class I and MHC -class II recognition events are capable generating a successful immune response to survivin antigen under appropriate circumstances, with the surprising result that cells that express survivin protein only internally, can in fact be identified by the immune system and killed.

According to the practice of the invention, there are provided liposomal compositions which upon administration will collect in the reticuloendothelial system, where a cell-mediated immune response to the antigen may be generated. Without being limited as to theory, it is believed that liposome degradation at such sites, causes antigenic survivin peptides to appear upon the surface of one or more types of cells capable of presenting , via major histocompatibility complex (MHC) -class I receptors to cytotoxic T-lymphocytes, or other subpopulations of T-cells, with the result that T cells come to recognize survivin in connection with MHC-I upon the surface of cells. Since MHC-I receptors are found on nearly all cell types, recognition of tumor cells expressing survivin is made possible. The present invention accordingly discloses that degradation of survivin within tumor cells causes sufficient survivin-related antigen to appear on the surface of such cells, for example in connection with cell surface MHC-class I, that T-cell recognition and killing of cancer cells is possible. In addition to such direct cell mediated immunity, anitbody mediated immune mechanisms also operate to inactivate tumor cells expressing survivin.

In an additional example of the invention, antibody-dependent cell mediated cytotoxicity is available to effect lysis of tumor cells that present survivin antigens on their surface. Again, while it is expected that most survivin is present within tumor cells, degradative processing lead to the presence on the surface of tumor cells of sufficient survivin antigens to attract antibodies with further recruitment of cytolytic cells. The presentation of such mechanisms is, as aforementioned, not a limitation upon the practice of the invention, but rather only to aid in the understanding of the novel clinical approaches of the invention, whose significance has not been

recognized. Additionally, the practitioner of the art will recognize that mechanisms exist for activation of certain classes of T-cells, such as T helper cells, following recognition of processed forms of antigen on the surface of antigen presenting cells, including macrophages, B-cells, and dendritic cells. Activated T helper cells, that is T-cells that have detected antigen presented with MHC-class II, may then contribute to B-cell activation/differentiation with the resultant production of antigen-specific antibody.

Preparation of survivin protein, or peptides thereof, as antigen

According to the practice of the invention, survivin, or peptide fragments thereof, are used to create vaccines which react with survivin, or peptide fragments thereof, as expressed intracellularly in tumor cells, and then presented to the immune system, for example in contact with surface MHC-class I glycoprotein. Survivin protein, and peptides thereof, may be prepared *in vitro* for administration to a subject's cells, or prepared *in situ*, that is, expressed directly from the subject's cells. Numerous methods for the expression of such constructs are described below. It is within the practice of the invention to use any of the expression methods described below, although it is recognized that such expression mechanisms are but representative of those known in the art. In a highly preferred example, survivin antigen is encapsulated in a liposome for delivery to the reticuloendothelial system where a cell-mediated immune response to the antigen may be generated.

Use of expression systems to generate survivin antigen

A wide variety of recombinant expression systems are available in order to generate survivin antigen for the purposes of administration to a subject in order to initiate an immune response. In connection with the use of such expression systems, the term survivin antigen includes any peptide fragments of survivin, to the extent that expression thereof is possible from the vector chosen as would be understood in the art. For example, conventional expression systems comprise an encoding DNA for survivin or for a peptide thereof (for example, generally for at least 30-40, preferably 40-50, more preferably 50-70, and most preferably, at least 70 amino acids thereof) operably linked to control systems such as promoters, terminating signals and the like.

Briefly, a preferred survivin antigen-encoding DNA, is expressed in any of a number of suitable recombinant expression vectors (the term "vector" being broadly construed to include plasmids and the like), such as those which may be derived, for example, from bacteria, yeast, insect cells, plant cells and mammalian cells. Antigen may also be expressed in such constructs as a fusion protein. Construction of such fusion proteins is common in the recombinant production of proteins, for example, in order to stabilize the product protein. It is noted that the fusion protein may contribute very usefully to the vaccine in that the additional heterologous amino acid sequence may confer immunogenicity-enhancing properties on the involved survivin epitopes. If however, the fusion protein is intended as an intermediate, it is useful to provide a cleavage site between the heterologous portion and the desired epitope. Such a cleavage site may represent a target sequence for a proteolytic enzyme or represent a methionine residue which may be cleaved by cyanogen bromide.

The following describe various expression systems which are available in the art for the production of quantities of survivin antigen useful in the practice of the invention. In connection with the use of such varied expression systems, it is recognized that the use thereof may lead to the production of glycoslyated or otherwise post-translationally modified forms of survivin that differ from survivin as expressed in human or other mammalian cells. Such differences in resultant expressed product may enhance the immunogenicity of survivin antigens, or detract from the usefulness thereof in the practice of the present invention. Should it be determined that any such difference is disadvantageous, an alternate expression system can simply be employed. Representative examples of useful expression systems include the following.

(a) Mammalian Systems

Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. Sequences encoding mammalian viral genes provide particularly useful promoter sequences. Additionally, the presence of an enhancer element combined with the promoter elements often increases expression levels. Typically, the survivin-

encoding sequence will also code for an N'- signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. Mammalian cell lines available as hosts for expression are well known in the art.

(b). Plant Cell Expression Systems

There are many plant cell culture and whole plant expression systems known in the art. Examples include those described in U.S. patents 5,693,506; US 5,659,122; and US 5,608,143. The use of plant cell signal peptides is also well known (see, for example, Vaulcombe et al., *Mol. Gen. Genet*· 209, pp. 33-40, 1987).

(c). Baculovirus Systems

The survivin encoding polynucleotide can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction may employ procedures known in the art. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987).

(d). Bacterial Systems

Bacterial expression techniques are also well recognized in the art. Generally it will be preferred to cause secretion of the survivin from the bacterial host, although collection of expressed proteins from inclusion bodies is also within the practice of the invention. Typcially the survivin encoding sequence will be maintained on a plasmid with a selection marker, and either a high or low copy number vector may be selected, depending upon the effect of the vector and the survivin protein on the host. Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector.

(e). Yeast expression systems

Yeast expression systems are well known in the art. DNA sequences that encode suitable signal sequences for secretion of survivin from yeast, include those described in EPO publication 012 873 and U.S. Patent 4,588,684. Additional related strategies for secretion are described in U.S. Patents 4,546,083 and 4,870,008.

Preparation of vectors for "gene therapy" to provide antigen *in situ*

With respect to survivin protein and peptide of sufficient length, antigen may be generated in situ, that is, in the cells of the subject, thereby permitting antigen presentation/immune recognition, for example mediated through MCH-class I, on the surface of cells that are modified to contain a suitable encoding DNA. Thus the expression vector may be considered the active ingredient in the vaccine. By "expression system" is meant any construct which is effective in producing the encoded protein in the desired target cells. The vaccine/vector may also include additional ingredients to target specific cells. Conventional expression systems comprise an encoding DNA for survivin or for a peptide thereof (for example, generally for at least 30-40, preferably 40-50, more preferably 50-70, and most preferably, at least 70 amino acids thereof) operably linked to control systems such as promoters, terminating signals and the like. Alternatively, the survivin or survivin peptide encoding nucleotides may be connected to encoding sequence for another protein, causing expression of a fusion protein, in which case requirements for the length of the survivin peptide sequence itself may be further relaxed. Additionally, as mentioned in greater detail below, various forms of "naked DNA" have recently been shown to be effective as expression systems in situ when injected into animals. The work of J. Ulmer, et al., *Science*, 259, pp. 1745-1749, and summarized by J. Cohen as a "Research News" presentation in the same issue at 1691-1692 has demonstrated this concept.

In effect, all such constructs provide a form of gene therapy, with the result that survivin is expressed, and immune response thereto can occur, for example when degraded survivin is presented in association with MHC-class I. Typically such vectors include additional DNA (such as to facilitate vector replication under independent conditions), and encapsulating material to facilitate entry into target cells. Examples thereof are as follows.

The gene delivery vehicle may be a viral vector and, more preferably, a retroviral, adenoviral, or adeno-associated viral (AAV). Additional viruses are recognized in the art as yielding constructs suitable for vector construction. See, for example, Jolly, *Cancer Gene Therapy*, 1, pp 51-64, 1994. See also RNA Tumor Viruses, Cold Spring Harbor Laboratory, 2nd edition, 1985. Retrovirus vectors can also be designed for site-specific integration into the chromosomes of a subject by

incorporation of a chimeric integrase enzyme into the retroviral particle (see WO96/37626). Packaging cell lines suitable for use with the retrovirus vectors are of course well known in the art and numerous examples of the use of retroviral vectors are described in the scientific and patent literature.

Human adenoviral gene therapy vectors are of use in the practice of the present invention. Exemplary known adenoviral gene therapy vectors employable in this invention include those described in WO 94/12649, WO 93/03769, WO 93/19191, WO 94/28938, WO 5/11984, WO 95/00655, WO 95/27071, WO 95/29993, and WO 95/34671.

Liposomes that can act as gene delivery vehicles are described in U.S. Patent No. 5,422,120, WO95/13796, WO94/23697, WO91/14445 and EP-524,968. Polycationic lipids, so called cationic amphiphiles, are also well recognized in the art as gene therapy vehicles (see for example WO 96/18372 and U.S. Patent 5,650,096). Additional polycationic agents that can be used to deliver polynucleotides to cells include histones, basic domains derived from transcription factors, spermine/spermidine polycations, and the like.

Naked DNA, that is, an encoding nucleotide sequence and sufficient 5' and 3' elements to direct expression therefrom, but not associated with lipid or viral delivery components, can also be used according to the practice of the invention. Examples of representative introduction methods are provided by U.S. Patent No. 5,580,859 and WO 90/11092. Uptake efficiency may be enhanced by use of biodegradable latex beads upon which the DNA is coated, through the mechanism of endocytosis.

Various organs of the body may be targeted by the above vectors. For example it is well established that naked DNA is efficiently expressed from muscle cells, and any killing of muscle cells expressing survivin antigen triggered by the induced immune responses thereto is expected to be acceptable in scope, particularly in view of the benefit derived. Other vectors may be targeted to various organs, and it is well recognized that many of the above-mentioned vectors exhibit tropisms for certain mammalian tissues which can be exploited in the practice of the present invention.

Preparation of antiidiotypic antibodies

An alternate approach to the practice of the invention is to prepare an "image" of the survivin antigen in the form of an antiidiotypic antibody. Ways to prepare both monoclonal and polyclonal antiidiotypic antibodies which bear the internal image of the tumor associated antigens are described, for example, in U.S. Patent No. 5, 053,224, the disclosure of which is incorporated by reference. Briefly, polyclonal antiidiotypic antibodies may be produced by immunizing animals with monoclonal idiotypic antibodies raised against the antigen and screened for reactivity with the antigen and screening for antisera which react with idiotypic antibodies to the survivin antigens. Monoclonal antibodies may also be prepared from such animals using standard techniques of immortalizing the antibody secreting cells of the animal and screening the cultures with idiotypic antibodies in competition with the survivin antigen. Human or murine monoclonals are preferred; polyclonal preparations made in a variety of mammalian systems may also be used.

Vaccine compositions

While the survivin antigens of the invention may by themselves constitute the vaccine, it is a further feature of the invention that these survivin antigens are administered in a formulation designed to enhance the antitumor response. Formulations include but are not limited to incorporation of the survivin antigen into a liposome with or without out additional adjuvants, use of adjuvants in general, and/or cloning a DNA that encodes survivin or peptide antigens thereof into a suitable vector, such as of viral or bacterial origin.

The formulations of the invention may only contain a single active ingredient - any combination of the immunogenic substances of the invention can be used. However, generally, such "cocktails" comprise active ingredients of the same type -- i.e., generally the active ingredient mixture will include either two or several antigens, two or several expression systems for protein or peptide antigens, or two or several antiidiotypic antibodies representing different antigens. However, there is no theoretical reason that, for example, a single vaccine could not include both antiidiotypic antibody and an expression system.

If the protein form of the antigen is to be used, it may be desirable to couple the protein or peptide to a carrier in order to enhance immunogenicity. Such coupling

can be effected using standard and conventional coupling techniques, optionally utilizing spacer moieties in order to provide correct juxtaposition of the carrier and epitope. A large number of suitable carriers for such purposes are known, including keyhole limpet hemocyanin, rotavirus VP6 inner capsid protein, pilin protein and the like. In addition, enhanced immunogenicity may be obtained by supplying the epitope or antigen in the form of a fusion protein wherein the epitope bearing portion is fused to heterologous amino acid sequences to enhance the effect of the epitope administered. Whether administered alone, coupled to carrier or as part of a fusion protein, the epitope bearing proteins of the invention, the DNA constructs, and the antiidiotypic antibodies are administered in the presence of suitable excipients. Conventional excipients may be used, but those mentioned herein are of particular interest.

Compositions employing liposomes encapsulating or conjugating to the active ingredient of the vaccine may be used and are especially preferred. Liposomes localize in the reticuloendothelial system, one of the sites of generation of the immune response in a mammalian host and enhance the immune response to antigens incorporated in the liposome. Macrophages, or other immune cells there present, are able to present survivin antigen in association with MHC-class I for immune system recognition. The liposomal formulations incorporating the survivin antigens may also include immune system adjuvants, including one or more of lipopolysaccharide (LPS), lipid A, or muramyl dipeptide (MDP) as described in Liposomes, Ostro MJ, Editor, Marcel Dekker, Inc. (1983) page 249). Other immune system adjuvants such as glucan or certain cytokines, including interleukins, interferons, and colony stimulating factors, such as IL1, IL2, gamma interferon, and GM-CSF may also be incorporated with antigen into the liposome.

The survivin antigen may also be formulated with various adjuvants which enhance antitumor response, in particular, cellular immune response to the survivin antigens. Such adjuvants include, but are not limited to, Freund's Complete Adjuvant, alum, lipid A, monophosphoryl lipid A, *Bacillus-Calmette-Guerrin* (BCG) and other bacteria, polysaccharides such as glucan, acemannan, and lentinan, saponins, detoxified endotoxin (DETOX), muramyl tripeptide, muramyl dipeptide and their derivatives, SAF1, lymphokines and cytokines, including interleukins and interferons

such as IL2 and gamma interferon, as well as colony stimulating factors such as GM-CSF, nonionic block copolymers, or immune stimulating complexes (ISCOMS).

Administration and Use

In the methods of the invention, the survivin cancer vaccine may be administered for both prevention and treatment of a wide variety of cancers. The survivin cancer vaccine of the invention is administered to subjects at risk for the development of cancer or showing an actual diagnosis thereof. While the target cancer may be specifically identified, the effect of the vaccines of the invention will be to enhance the potential of the immune system generally, generating T cell responses as well as the production of antibodies. To the extent that the enhancement of the cellular immune system is effected, the vaccines of the invention are useful in the prevention and therapy of other types of cancer as well as that which may have been specifically diagnosed. Thus, the cellular responses and humoral responses generated according to the practice of the invention are effective against, for example, cancers of the colon, lung, bladder, stomach, breast, cervix, and the like as well as lymphomas and leukemias.

The compositions are formulated for parenteral administration, for example, using a formulation appropriate to the administration route such as those described in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company, Easton, PA.

Suitable routes for parenteral administration include intracutaneous, subcutaneous, intramuscular, and intravenous injection or oral administration. For formulation for injection, the vaccine is generally formulated in a suitable liquid such as Hank's solution or Ringer's solution along with suitable excipients providing buffering, stabilizing, and other desirable characteristics, as well as additional components desired, as described below. Alternative routes for parenteral administration include oral administration in which case additional components for stabilizing the preparation may also be included.

In addition to administration in an appropriate isotonic vehicle for injection, liposomes are desirably used as a carrier to direct the product to the immune system as disclosed in copending application 07/800,474, the disclosure of which is

incorporated herein by reference. As described further in Example 1 below, the disclosure of U.S. application 09/086,552 filed May 28, 1998 is also specifically incorporated herein by reference.

In general, the dosage range for the survivin antigens of the invention is of the order of 0.01 μg-100 mg per dose, preferably 0.1 μg-10 mg per dose and more preferably 10 μg-1 mg per dose. Suitable volumes for parenteral administration are about 0.1-5 ml. It is recognized that such recommended doses may vary from application to application, and from patient to patient, but can be readily determined by those in the medical art.

The protocols may involve administration of cocktails of various antigens or their idiotypic representatives or may involve sequential administration of these active ingredients. The antigens and their representatives may represent a variety of immunogens or may represent different forms of the same immunogen. In general, protocols involving one or more immunogenic species can be designed according to routine optimization procedures. Particular protocols of administration will vary from treatment to treatment according to medical practice, as is to be expected given the wide range of tumors that can be treated according to the practice of the present invention.

In addition, it may be advantageous to substitute, for a first administration, a recombinant form of the antigen wherein the antigen gene or cDNA is administered in a living expression vector such as vaccinia virus.

## Examples

### Example 1: Preparation of Optimized Liposome Formulations

As aforementioned, the use of liposomes to deliver survivin to a subject represents a preferred embodiment of the invention. Numerous examples of such preparations, useful in the practice of the present invention, are described in U.S. patent application 09/086,552 which is incorporated by reference herein. Certain preferred embodiments are prepared as follows.

Liposomes are prepared according to the procedure of Alving, C.R. et al. in Liposome Technology: Interactions of Liposomes with the Biological Milieu (1993) III: CRC Press, Boca Raton, FL, pp. 317-343. Additional descriptions are found in

Verma, J.N. *et al. Infect Immun* (1992) 60:2438-2444, and in Richards, R.L. *et al. Infect Immun* (1988) 56:682-686. Table 1 below shows abbreviations used for the components that may be employed.

**Table 1**

| Abbrev. | Name | No. Carbons in each acyl | No. π-bonds in each acyl |
|---|---|---|---|
| DLPC | dilauroyl phosphatidyl-choline | 12 | 0 |
| DMPC | dimyristoyl phosphatidyl-choline | 14 | 0 |
| DPPC | dipalitoyl phosphatidyl-choline | 16 | 0 |
| DSPC | distearoyl phosphatidyl-choline | 18 | 0 |
| DOPC | dioleoyl phosphatidyl-choline | 18 | 1 |
| DLnPC | dilinoleoyl phosphatidyl-choline | 18 | 2 |
| DMPG | dimyristoyl phosphatidyl-glycerol | 14 | 0 |
| CHOL | cholesterol | | |
| LA | Lipid A | | |

In a typical preparation, multilamellar liposomes are made from a mixture of DMPC:DMPG:CHOL:LA in a molar ratio of 9:1:7.5:0.011. The lipid A is included as an adjuvant. The lipid mixture is rotary evaporated to a dry thin film at approximately 40°C *in vacuo* from a chloroform solution in a pear-shaped flask. To ensure complete removal of the organic solvent, the flask is then dried under very low vacuum (about 0.05 mm Hg) overnight in a desiccator at room temperature. After drying, the lipids are carefully swollen in deionized, sterile pyrogen-free water by vortexing. The resulting suspension is frozen at -55°C, lyophilized at -20°C overnight and 0°C-10°C the following day using the Virtis Unitop 800SL Freeze Mobile (the Virtis Company, Gardener, NY).

The lyophilized lipids are then reconstituted in the presence of the substance to be encapsulated (i.e. survivin antigen) to obtain multilamellar liposomes containing this substance. A suitable reconstituting buffer is phosphate-buffered saline (PBS) or

Tris-glycine/NaCl. The liposomal phospholipid concentration in the reconstituting buffer is 10-200 mM.

Unencapsulated substance may be removed by washing the liposomes three times with 0.15 M NaCl at 27000 x g for 10 minutes at 10°C. The resulting liposomes are suspended either in 0.15 M NaCl or an appropriate isotonic buffer to reach a final phospholipid concentration of 10-200 mM. Alternatively, the wash step may be omitted, leaving both unencapsulated and encapsulated antigen in the preparation. However, inclusion of a wash step is preferred.

Effect of liposomes on emulsion stability

An oil-in-water emulsion was prepared from mineral oil using the method of Rudbach, J.A. *et al.*, Adjuvants: Theory and Practical Applications (Stewart-Tull, Ed., John Wiley & Sons, New York, in press) or Schneerson, R. *et al. J Immunol* (1991) 147:2136. Various amounts of a liposome preparation composed of DMPC:DMPG:CHOL:LA (9:1:7.5:0.11) were added to the emulsified o/w composition which contained no other stabilizer. The stabilizing effects of various amounts of the liposome composition were determined. Stability (or instability) was determined as percent separation after five days at 37°C as a function of the concentration of liposomal phospholipids. It was determined that when the liposomal phospholipid concentration reached 100 mM or more, substantial stabilization of the preparation was obtained. The emulsion appears essentially completely stable after this time at this temperature when the liposomal phospholipid concentration is 125 mM or greater.

These findings are in contrast to the effect of liposomes on a "stabilized" water-in-oil (w/o) emulsion. Incomplete Freund's Adjuvant, stabilized with Arlacel A, was supplemented with 0%, 5%, 20% and 50% liposomes and incubated at 4°C for 15 days. The percent separation at these concentrations was 1.3%, 5.71%, 20% and 40%, respectively. Thus, the result for o/w compositions upon addition of liposomes was markedly different from the water-in-oil counterparts.

The kinetics of stabilization of liposomes on mineral oil-in-water emulsions prepared by a syringe extrusion procedure was also tested at various concentrations of liposomes prepared as described above. Results were obtained when the emulsions

were incubated at various concentrations of liposomes at 37°C. As determined, liposomal concentrations providing 125 mM phospholipid or more gave o/w emulsions that were stable to the level of detection for at least five days. The addition of liposomes corresponding to 100 mM phospholipid resulted in stabilizing the emulsion at a level of 40% separation which was reached after approximately one day. However, smaller amounts of liposomes failed to stabilize the emulsion, which showed 100% separation in less than a day.

Results obtained when the oil-in-water emulsions were prepared by a vortex mixing procedure differed from those obtained from syringe mixing. While again, 125 mM phospholipids provided by liposomes showed a stabilizing effect, this was dramatically less than for the emulsions prepared by the syringe extrusion procedure, and for far shorter times. The results obtained with 100 mM phospholipid were similar to those obtained with 125 mM phospholipid. Interestingly, with higher amounts, at 150 mM phospholipid, the emulsion appeared less stable and began to separate almost immediately. At amounts lower than 100 mM phospholipid, separation began immediately and was almost complete before the end of one day, as was the case with the syringe extrusion procedure.

Effect of the Nature of the Oil Components

The experiments set forth above were repeated using various oils in the o/w emulsion, including N-dodecane, N-hexadecane, squalane, squalene, corn oil, and peanut oil. In all cases, again, the addition of liposomes sufficient to provide 125 mM phospholipid resulted in emulsions with no detectable separation after five days. In these experiments liposomes sufficient to provide 100 mM phospholipid also provided this result. However, lower phospholipid concentrations failed to stabilize the emulsions which separated in all cases over a period of one day or less.

## Claims

1. A vaccine for eliciting an antitumor immune response in a subject which comprises an active ingredient selected from the group consisting of:

(a) an antigen that comprises one or more epitopes of survivin protein;

(b) a recombinant expression system capable of expressing survivin antigen for administration to the tissues of said subject;

(c) a DNA molecule that comprises an encoding sequence for survivin antigen, capable of expression of said antigen in the cells of a subject; and

(d) an antiidiotypic antibody, or fragment thereof, which mimics said antigen.

2. The vaccine of claim 1 which further comprises at least one adjuvant capable of enhancing said antitumor immune response.

3. The vaccine of claim 1 wherein the antigen is encapsulated in, or coupled to, a liposome.

4. The vaccine of claim 1 that comprises one or more epitopes of survivin protein.

5. The vaccine of claim 1 that comprises a recombinant expression system capable of expressing survivin antigen for administration to the tissues of said subject.

6. The vaccine of claim 1 comprising a DNA molecule, said DNA molecule comprising an encoding sequence for survivin antigen, capable of expression of said antigen in the cells of a subject.

7. The vaccine of claim 1 that comprises an antiidiotypic antibody, or fragment thereof, which mimics said antigen.

8. A method to induce an antitumor immune response in a potential or tumor-bearing subject, which method comprises administering to said subject the vaccine of claim 1.

9. The method of claim 8 wherein said subject has been treated to excise said tumor, but is at risk for recurrence thereof.

10. The method of claim 8 wherein said subject is a human patient.

11. The method of claim 8 wherein said subject is a veterinary patient.

# INTERNATIONAL SEARCH REPORT

Intr tional Application No
PC I/US 99/15832

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 A61K39/00 A61K48/00 A61K39/395 A61K39/39
//C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 98 22589 A (UNIV YALE ;ALTIERI DARIO C (US)) 28 May 1998 (1998-05-28) cited in the application example 15 page 28, line 5 -page 29, line 2 --- | 1,2,4,5, 8-11 |
| A | WO 95 04548 A (JENNER TECHNOLOGIES) 16 February 1995 (1995-02-16) page 1, line 5-10 page 3, line 27-35 page 11, line 11-22 --- | 1-11 |
| A | ADIDA, C. ET AL: "Anti-apoptosis gene, survivin, and prognosis of neuroblastoma" LANCET, (19980321) VOL. 351, NO. 9106, PP. 882-883. ISSN: 0099-5355., XP002120397 the whole document --- -/-- | 1-11 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 26 October 1999 | 09/11/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016 | Covone, M |

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Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Intr tional Application No
PCI/US 99/15832

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | ANONYMOUS: "New gene product prevents apoptosis in colon, pancreas cancers 'news!." GASTROENTEROLOGY, (1997 OCT) 113 (4) 1060. , XP002120398 the whole document | 1-11 |
| P,X | WO 99 13073 A (HAMADA HIROFUMI ;RPR GENCELL ASIA PACIFIC INC (JP)) 18 March 1999 (1999-03-18) page 2, line 30 -page 3, line 9 page 5, line 29-31 page 24, line 27 -page 25, line 4 | 1,4-6, 8-11 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

rnational application No.
PCT/US 99/ 15832

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 8-11
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant. this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee. this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid. specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 8-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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Continuation of Box I.1

Claims Nos.: 8-11

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

# INTERNATIONAL SEARCH REPORT

nformation on patent family members

Inte tional Application No
PCI/US 99/15832

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9822589 A | 28-05-1998 | AU 7301898 A | 10-06-1998 |
| | | EP 0950103 A | 20-10-1999 |
| WO 9504548 A | 16-02-1995 | AU 686660 B | 12-02-1998 |
| | | AU 7631294 A | 28-02-1995 |
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| | | EP 0721345 A | 17-07-1996 |
| | | JP 9504000 T | 22-04-1997 |
| | | US 5925362 A | 20-07-1999 |
| WO 9913073 A | 18-03-1999 | JP 11075859 A | 23-03-1999 |
| | | AU 8999198 A | 29-03-1999 |